Life Sciences 118 (2014) 288–296

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Endothelin receptor antagonist exacerbates autoimmune myocarditis in mice

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ARTICLE INFO

Article history:
Received 15 October 2013
Accepted 8 January 2014
Available online 18 January 2014

Keywords:
Autoimmune myocarditis
Endothelin
Suppressor of cytokine signaling
Inflammation

ABSTRACT

Aims: Myocarditis and subsequent dilated cardiomyopathy are major causes of heart failure in young adults. Experimental autoimmune myocarditis (EAM) is a mouse model of post-infectious myocarditis and inflammatory cardiomyopathy. The pathological role of endothelin (ET) in myocarditis has not been elucidated.

Main methods: EAM was induced by immunization of cardiac myosin peptide with complete Freund's adjuvant on days 0 and 7 in BALB/c mice. An ET_A/ET_B dual receptor antagonist, SB209670, was administered by a continuous infusion from a subcutaneous pump for 2 weeks.

Key findings: An increase in the heart-to-body weight ratio was observed in SB209670-treated mice compared with vehicle-treated mice. Heart pathology in SB209670-treated mice was remarkable for gross inflammatory infiltration, in contrast to the lesser inflammation in the hearts of vehicle-treated mice. We found that an ET blockade decreased the number of $Foxp3^+$ regulatory T cells in the heart. The ET blockade also inhibited the expression of the suppressor of cytokine signaling 3 that plays a key role in the negative regulation of both Toll-like receptor- and cytokine receptor-mediated signaling. EAM is a $CD4^+$ T cell-mediated disease. $CD4^+$ T cells isolated from SB209670-treated EAM mice produced less IL-10 and more inflammatory cytokines, IL-6 and IL-17, than those isolated from vehicle-treated mice.

Significance: The ET receptor antagonist exacerbated autoimmune myocarditis in mice. Our novel findings suggest that ET may play an important role in the regulation of inflammation in myocarditis.

Introduction

Myocarditis is an inflammatory disease of the myocardium and one of the leading causes of dilated cardiomyopathy (DCM), which is currently the most frequent reason for heart transplantation (Kindermann et al., 2012). Viral infections are thought to be the most common primary cause of myocarditis in Western countries (Leuschner et al., 2009). Although its pathogenesis remains unclear, there is substantial evidence that a post-viral autoimmune-mediated response to cardiac antigens critically contributes to the development and progression of myocarditis (Kindermann et al., 2012; Lazzerini et al., 2013). On this basis, two animal models of experimental myocarditis have been used and have greatly advanced our knowledge of the pathogenesis of the disease; virus-induced myocarditis and experimental autoimmune myocarditis (EAM) (Lazzerini et al., 2013).

Endothelin (ET) has emerged as an important participant in the pathophysiology of a variety of cardiovascular diseases (Barton et al., 1998; Murakoshi et al., 2002; Sakai et al., 1996; Stewart et al., 1991). Experimental and clinical studies have demonstrated that the activation of the ET system in many diseases is characterized by inflammation or fibrotic remodeling. Several ET antagonists have been discovered, which has helped elucidate the mechanisms by which ET mediates its effects. There are a few publications on the effects of ET receptor blockers on virus-induced myocarditis models (Marchant et al., 2009; Ono et al., 1999; Seta et al., 2000), and the efficacy of an ET receptor blockade on viral myocarditis is still controversial. In a mouse model of Coxsackievirus B3 (CVB3)-induced myocarditis, Bosentan, an ET_A/ET_B dual receptor blocker, improved cardiac function, but enhanced the viral load and myocarditis severity through ET_A receptor mediated p38 MAPK activation (Marchant et al., 2009). Thus, antagonism of ET signaling is potentially a desirable therapeutic strategy for cardiac dysfunction; however it is still unclear whether an ET blockade is useful for controlling the inflammation itself or not.

To answer this question, we evaluated the effect of an ET receptor blockade on EAM in mice. EAM was induced by immunization with cardiac myosin peptide, which allowed us to examine the effect of ET receptor blockers on myocardial inflammation under infectious pathogen-free conditions.

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http://dx.doi.org/10.1016/j.lfs.2014.01.007

Materials and methods

Study approval

All animal experiments were approved by the Institutional Animal Experiment Committee of the University of Tsukuba.

Mice

BALB/c mice were purchased from CLEA Japan. We used 6- to 8-week-old male mice.

Immunization protocol

The mice were immunized with 100 μg of murine cardiac α-myosin heavy chain (MyHC-α) peptide (MyHC-$\alpha_{614-629}$ [Ac-RSLKLMATLFSTYASADR-OH]; Toray Research Center) emulsified 1:1 in phosphate buffered saline (PBS)/complete Freund's adjuvant (CFA) (1 mg/ml; H37Ra; Sigma-Aldrich) on days 0 and 7 as described previously (Sonderegger et al., 2008; Tajiri et al., 2012, 2013).

Treatment

The mice were given either an ET_A/ET_B dual receptor antagonist, SB209670 [(+)-(1S,2R,3S)-3-(2-carboxymethoxy4-methoxyphenyl)-1-(3,4-methylenedioxyphenyl)-5-(prop-1-yloxy)indane-2-carboxylic acid] (n = 15) (10 mg/kg/day: SmithKline Beecham Pharmaceuticals), or saline (n = 15) (vehicle), subcutaneously using an ALZET® mini-osmotic pump (Model 2002, DURECT Corporation), implanted in the back. We previously investigated the effect of SB209670 on the development of hypertension, cardiac hypertrophy, glomerulosclerosis, and renal vascular wall thickening in transgenic hypertensive mice with over-expression of both human renin and angiotensinogen genes (Maki et al., 2004). The dose of the S209670 (10 mg/kg/day) was confirmed to be adequate to completely inhibit the effects of the ET-1 administered in the mice. The mice were treated for 2 weeks from day 0 to day 14 after immunization and sacrificed on day 14 for further analyses.

Histopathological examination

Myocarditis severity was scored on hematoxylin and eosin (H&E)-stained sections using grades from 0 to 4: 0, no inflammation; 1, less than 25% of the heart section involved; 2, 25 to 50%; 3, 50 to 75%; and 4, more than 75% as described previously (Sonderegger et al., 2008; Tajiri et al., 2012, 2013). Two independent researchers scored the slides separately in a blinded manner.

Flow cytometric analyses and intracellular cytokine staining

Heart inflammatory cells were isolated and processed as previously described (Eriksson et al., 2003; Valaperti et al., 2008). For the flow cytometric analysis of the surface markers and cytoplasmic cytokines, the cells were stained directly with conjugated fluorescence antibodies and analyzed with a FACSCalibur instrument (BD Biosciences). For the analysis of the intracellular cytokine production, the cells were stimulated with 50 ng/ml of phorbol 12–myristate 13–acetate (PMA), 750 ng/ml of ionomycin (Sigma-Aldrich) and 10 μg/ml of brefeldin A (eBioscience) for 5 h. The fluorochrome-conjugated, mouse-specific monoclonal antibodies purchased from eBioscience, included CD4 and forkhead box P3 (Foxp3).

Cytokine ELISA

For the analysis of the cytokines and chemokines in the heart, the hearts were homogenized in media containing 2.5% fetal bovine serum. The supernatants were collected after centrifugation and stored at −80 °C. The concentrations of cytokines and chemokines in the heart homogenates and culture supernatants were measured with Quantikine ELISA kits (R&D Systems).

Isolation of dendritic cells (DCs) and $CD4^+$ T cells

We used magnetic-activated cell sorting kits for the cell isolation (CD11c MicroBeads for DC isolation, $CD4^+CD62L^+$ T Cell Isolation Kit II for naïve $CD4^+$ T-cell isolation and $CD4^+$ T Cell Isolation Kit II for $CD4^+$ T-cell isolation, Miltenyi Biotec).

In vitro T-cell differentiation

Purified naïve $CD4^+CD62L^+$ T cells were stimulated with anti-CD3 1 μg/ml (R&D Systems) and anti-CD28 1 μg/ml (Acris Antibodies), with or without ET-1 under T helper (Th)1-, Th2-, Th17- or Treg polarizing conditions for 48 h. Th1 condition: IL-12 (10 ng/ml) and anti-IL-4 antibody (10 μg/ml). Th2 condition: IL-4 (10 ng/ml), anti-IL-12 (10 μg/ml), and anti-IFN-γ (10 μg/ml). Th17 condition: transforming growth factor (TGF)-β (10 ng/ml), IL-6 (20 ng/ml), IL-23 (20 ng/ml), anti-IL-4 (10 μg/ml), anti-IL-12 (10 μg/ml) and anti-IFN-γ (10 μg/ml). Treg condition: TGFβ1 (10 ng/ml), anti-IL-4 (10 μg/ml), anti-IL-12 (10 μg/ml), and anti-IFN-γ (10 μg/ml). The cytokines and antibodies were obtained from the R&D Systems except for the TGF-β (BioLegend).

Quantitative real-time reverse transcription polymerase chain reaction (QRT-PCR)

The total RNA was prepared using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The cDNA was synthesized from 1 μg of the total RNA by reverse transcriptase (Takara). QRT-PCR analysis was performed with LightCycler (Roche Diagnostics). The oligonucleotides used for the PCR amplification were the following: *Ece1* forward, TGGAGGTTATGTATGGGACGA; *Ece1* reverse, GGTTGTTTTCCG TGTCACTCA; *Edn1* forward, TCCTTGATGGACAAGGAGTGT; *Edn1* reverse, CCCAGTCCATACGGTACGA; *Edn2* forward, AGACCTCCTCCGAAAGCTG; *Edn2* reverse, TTTCTTGTCACCTCTGGCTGTA; *Edn3* forward, GCACCAGA GATGTCACCAGTT; *Edn3* reverse, AGTCTCCCGCATCTCTTCTG; *Ednra* forward, TGTGAGCAAGAAATTCAAAAATTG; *Ednra* reverse, ATGAGGCTTT TGGACTGGTG; *Ednrb* forward, TAGAGGCAACCGGGCTAGT; *Ednrb* reverse, GGGGAGTGAAGACAGGACAC; *Ifng* forward, ATCTGGAGGAACTG GCAAAA; *Ifng* reverse, TTCAAGACTTCAAAGAGTCTGAGGTA; *Il4* forward, CATCGGCATTTTGAACGAG; *Il4* reverse, CGAGCTCACTCTCTGTGGTG; *Il17a* forward, TGTGAAGGTCAACCTCAAAGTCT; *Il17a* reverse, GAGGGA TATCTATCAGGGTCTTCAT; *Socs3* forward, ATTTCGCTTCGGGACTAGC; *Socs3* reverse, AACTTGCTGTGGGTGACCAT; *Hprt* forward, TCCTCCTCAG ACCGCTTTT; and *Hprt* reverse CCTGGTTCATCATCGCTAATC. The data were normalized by the level of the *Hprt* expression in each sample.

Statistical analysis

Statistical analyses were performed using the two-tailed *t* test or Mann–Whitney *U* test, for experiments comparing two groups. For multiple comparisons, one-way analysis of variance with Dunnett's post-hoc test was used. *P* values < 0.05 were considered statistically significant.

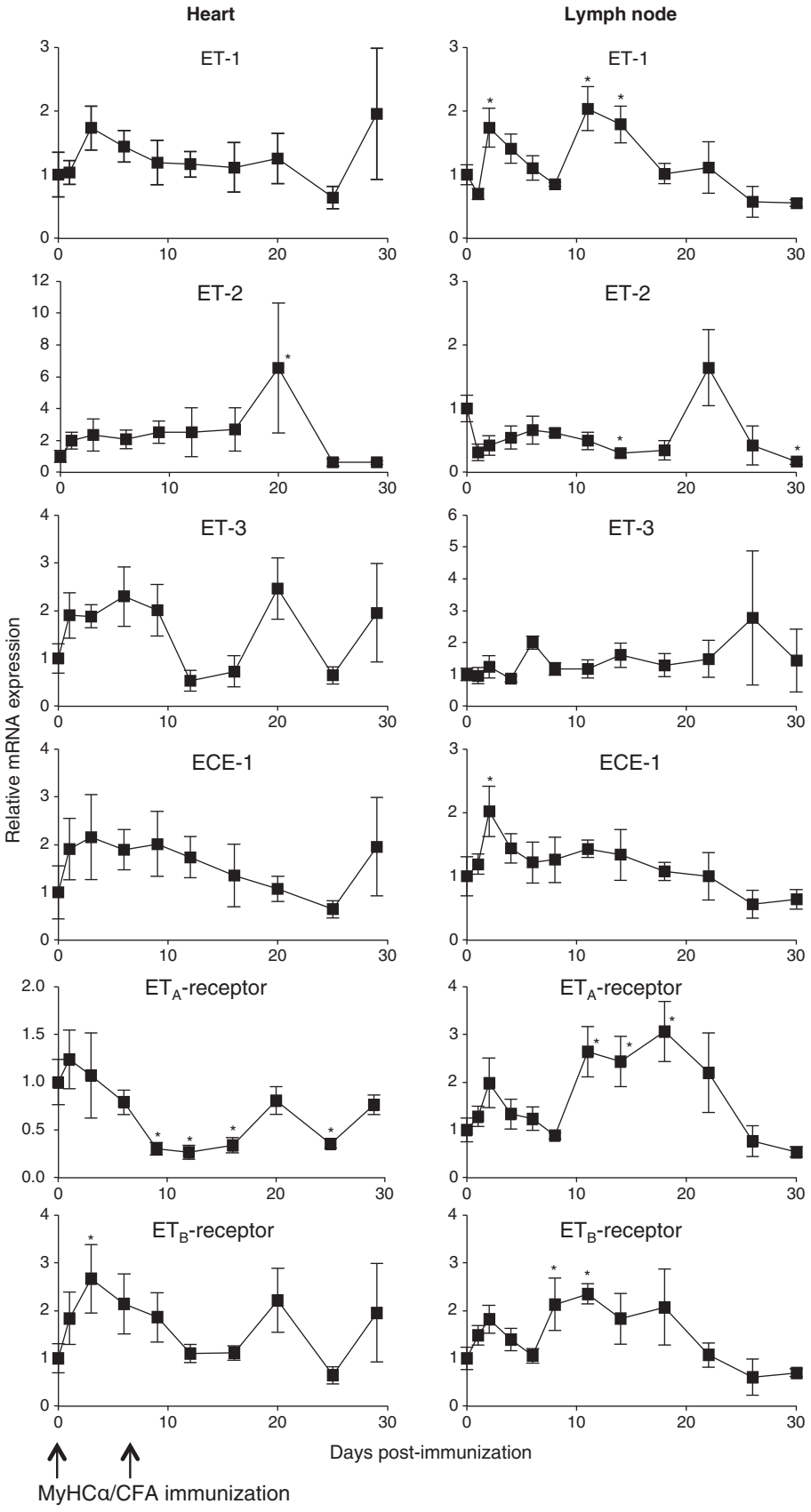

Fig. 1. The expression of ET-related genes in the EAM hearts and draining lymph nodes. EAM was induced by a cardiac myosin peptide immunization on days 0 and 7. We obtained hearts (n = 5 mice each day) and draining lymph nodes (n = 4 mice each day) at days 0, 1, 3, 6, 9, 12, 16, 20, 25 and 29, and extracted the mRNA from them. The expression of the ET-related genes was quantified by QRT-PCR. The results of one of two representative experiments are shown. The data were normalized by the basal gene expression (day 0). Data are expressed as the mean ± SEM. *$P < 0.05$ vs. day 0.

Results

The expression of ET-related genes during the course of EAM

First, we checked the expression of ET-related genes in the EAM hearts and the draining lymph nodes (Fig. 1). The ET-1 was elevated in the early phase especially in the lymph nodes. On the other hand, the ET-2 was elevated in the chronic phase. The ET-3 level did not change significantly throughout the disease course. The endothelin converting enzyme-1 (ECE-1) was upregulated in the very early phase in the lymph nodes. Interestingly, the ET_A-receptor expression was downregulated in the heart, but upregulated in the lymph nodes. The ET_B-receptor was upregulated in the heart and lymph nodes in the early phase. Thus, the ET-related gene expression changed in the course of the disease. However, the pathological role of the ET system in myocarditis has not been elucidated.

An ET_A/ET_B dual receptor blocker exacerbates autoimmune myocarditis

Next, we sought to determine the in vivo effects of the ET receptor blockade on EAM. As shown in Fig. 1, the ET-1 gene expression elevated immediately after the 1st immunization in the lymph nodes, and the peak of the inflammatory response in the heart occurred on day 14 in this EAM model. Therefore we administered an ET_A/ET_B dual receptor antagonist, SB209670, by a continuous infusion with a subcutaneous pump from day 0 to day 14, and the mice were sacrificed on day 14. As shown in Fig. 2A, the hearts from the SB209670-treated mice had severe inflammation with mononuclear cell infiltration with a higher myocarditis severity score (Fig. 2B). The heart-to-body weight ratio in the SB209670-treated mice was significantly increased compared to that in the control mice (Fig. 2C). We also examined whether the SB209670 treatment had an effect on the cytokine and chemokine milieu in the heart. On day 14 after the MyHC-α immunization, the heart

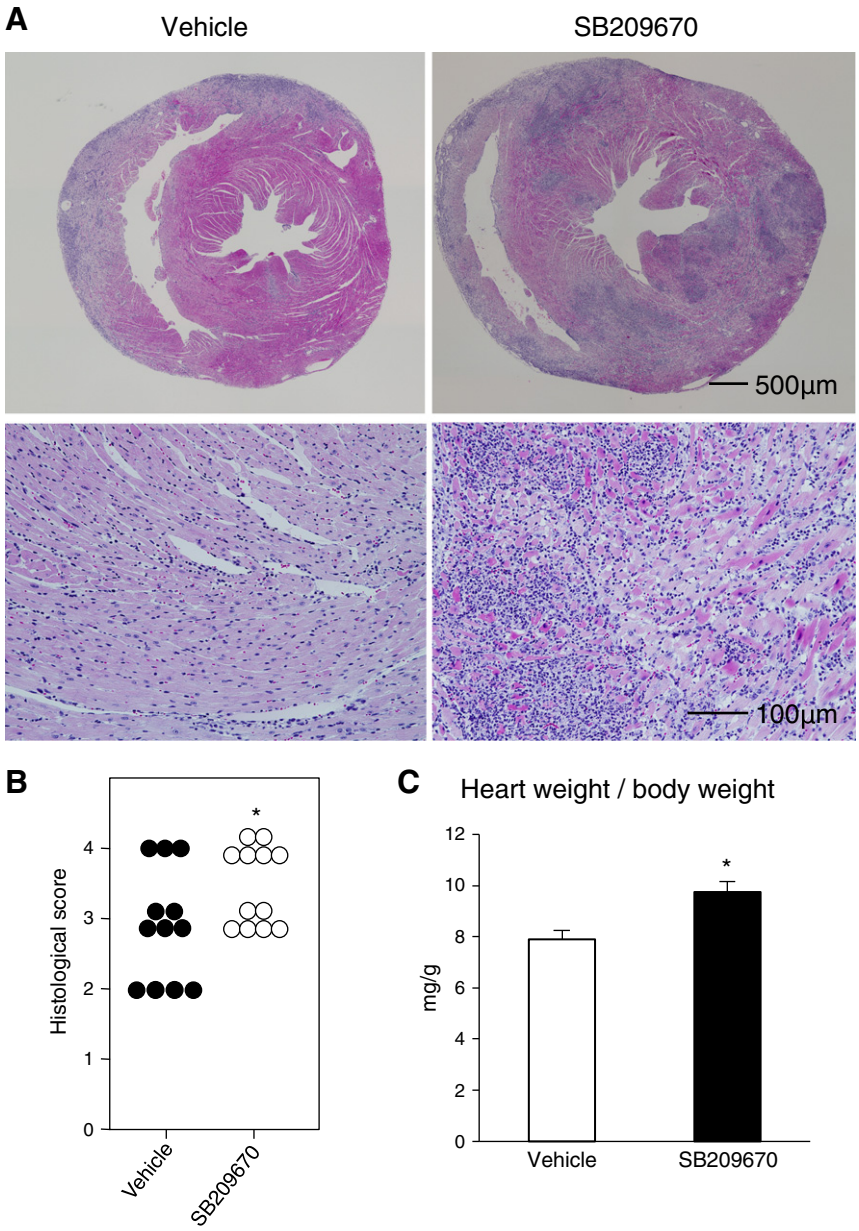

Fig. 2. ET_A/ET_B dual receptor blocker exacerbates autoimmune myocarditis. EAM mice were treated with SB209670 (10 mg/kg/day) or a vehicle (saline) for 2 weeks from day 0 to day 14 after immunization. (A) Representative H&E-stained sections of the hearts. Scale bars, 500 or 100 μm. (B) Myocarditis severity in the heart sections (n = 12 per group). (C) Heart-to-body weight ratios of control and SB209670-treated mice (n = 7 per group). The results of one of two representative experiments are shown. Data are expressed as the mean ± SEM. *$P < 0.05$.

homogenates from SB209670-treated mice had significantly increased amounts of proinflammatory cytokines, including IL-1β and IL-17A, and chemokines, including chemokine (C–C motif) ligand (CCL)3 and CCL5 and chemokine (C–X–C motif) ligand (CXCL)1 (Fig. 3). Thus, an ET receptor blocker exacerbated the EAM development, which corresponded to an increase in the proinflammatory cytokines and chemokines in the heart.

ET receptor antagonist downregulated suppressor of cytokine signaling (SOCS)3 gene expression

SOCS proteins are negative regulators of cytokine signaling (Dimitriou et al., 2008; Yoshimura et al., 2007). SOCS3 is a key negative-feedback regulator of gp130, which is a common receptor component of the IL-6 cytokine family (Yajima et al., 2011). The major function of SOCS3 is inhibition of signaling by the IL-6 family of cytokines, causing inhibition of STAT3 activation (Yoshimura et al., 2007). In the early phase of EAM, the SOCS3 gene expression was upregulated in the draining lymph nodes and hearts (Fig. 4), and then downregulated, coincident with disease remission.

We then examined the effect of SB209670 treatment on the gene expression of SOCS3, and found that it was downregulated on day 14 in the hearts (Fig. 5A). It has recently been reported that SOCS3 in the antigen-presenting cells (APCs), e.g., DCs and macrophages, regulates inflammation and protects against detrimental inflammatory responses (Li et al., 2006; Qin et al., 2012). To examine the effect of the ET-1 on the SOCS3 gene expression in DCs, we isolated the DCs from healthy mice and stimulated them with ET-1 at various concentrations. As shown in Fig. 5B, the SOCS3 gene expression was upregulated in a dose-dependent manner by the ET-1 stimulation. We also found that an ET_A-receptor antagonist, BQ-123, but not an ET_B-receptor antagonist, A-192621, blocked the ET-1-altered SOCS3 gene expression (Fig. 5C). These results suggest that ET-1–ET_A-receptor signaling may increase the SOCS3 gene expression in the DCs, which provides protection from myocarditis through deactivation of inflammatory responses.

Cardiac myosine-specific $CD4^+$ T cell response and cytokine production

EAM is a $CD4^+$ T cell-mediated disease (Eriksson et al., 2003; Fairweather et al., 2001). To evaluate the myosin-specific T-cell function, the $CD4^+$ T cells were isolated from myocarditis mice and restimulated with a myosin peptide, and the cytokines in the culture supernatant were analyzed by ELISA. The $CD4^+$ T cells from SB209670-treated mice produced a much greater amount of proinflammatory cytokines, such as IL-6, IL-17 and TNF-α, but less IL-10, which is an anti-inflammatory cytokine (Fig. 6). Next, we analyzed the number of regulatory T cells (Tregs) in the heart by using flow cytometry. The

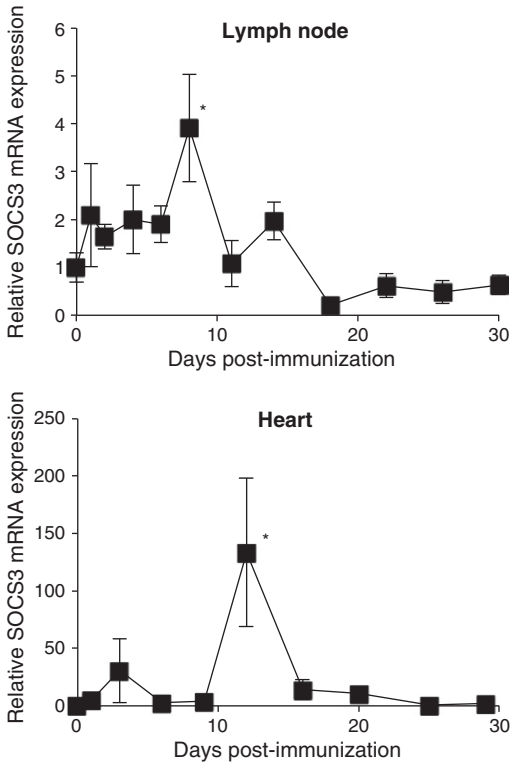

Fig. 4. The SOCS3 gene expression in the EAM hearts and draining lymph nodes. The hearts (n = 5 mice each day) and draining lymph nodes (n = 4 mice each day) were harvested at days 0, 1, 3, 6, 9, 12, 16, 20, 25 and 29, and we extracted the mRNA from them. The SOCS3 gene expression was quantified by QRT-PCR. The results of one of two representative experiments are shown. The data were normalized by the basal gene expression (day 0). Data are expressed as the mean ± SEM. *$P < 0.05$ vs. day 0.

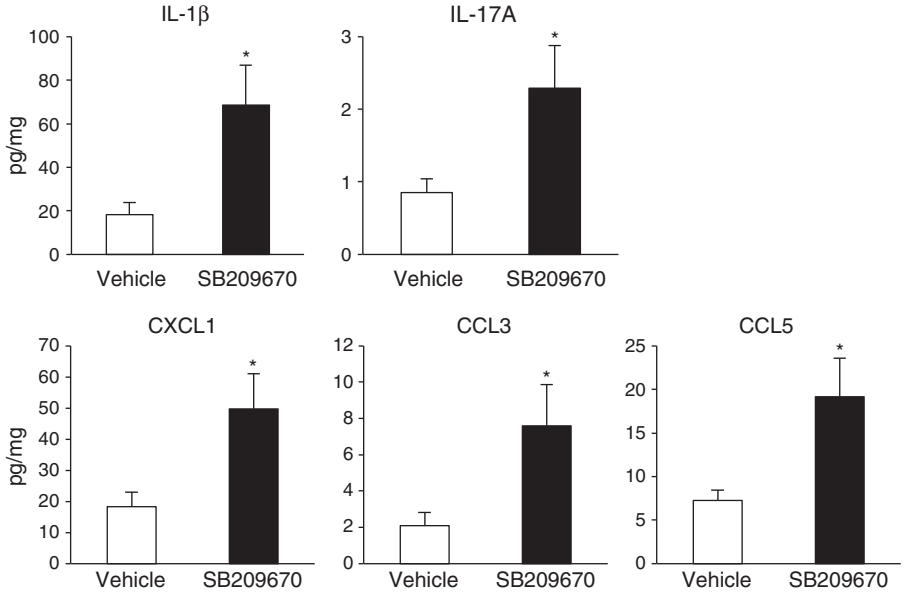

Fig. 3. The production of cytokines and chemokines in the hearts. Myocardial tissues from SB209670- and vehicle-treated EAM mice were homogenated and processed by ELISA to detect the cytokines and chemokines on day 14. The bar graphs show the group means ± SEM of 5 mice per group. The results of one of two representative experiments are shown. *$P < 0.05$.

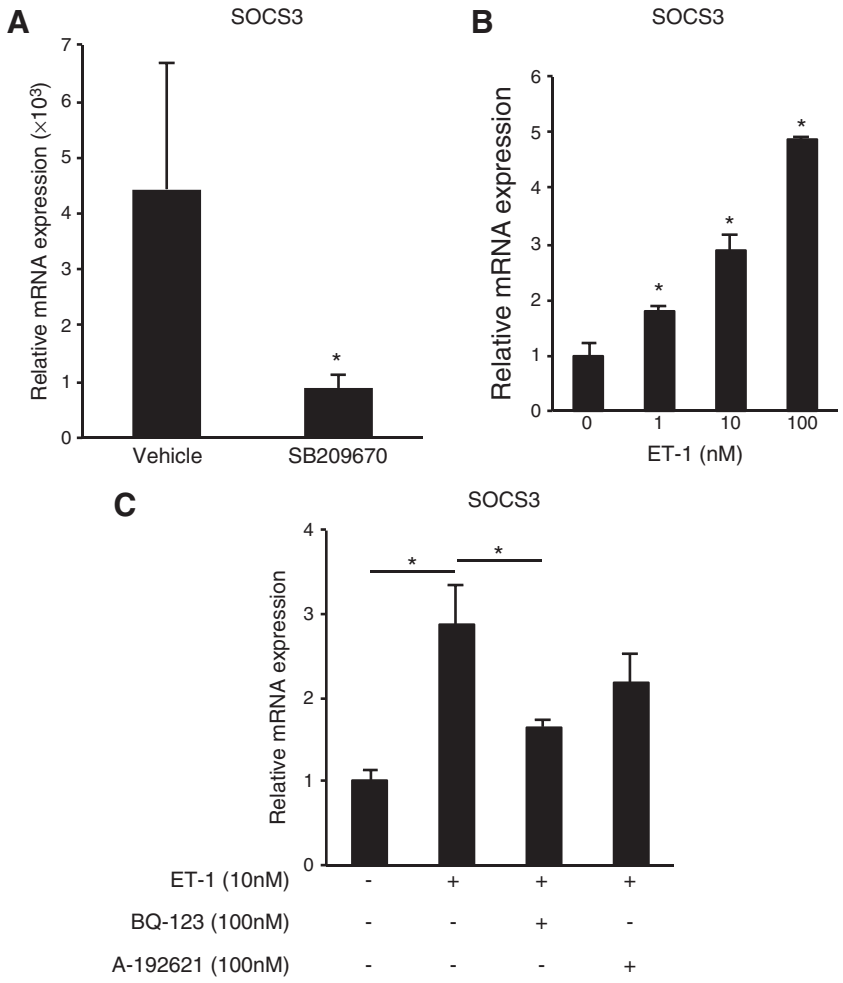

Fig. 5. ET receptor antagonists downregulate SOCS3 gene expression. (A) The SOCS3 gene expression in the hearts on day 14 was quantified by QRT-PCR (n = 6 per group). (B) DCs from healthy mice were stimulated with ET-1 at various concentrations. The SOCS3 gene expression was quantified by QRT-PCR. (C) An ET_A-receptor antagonist, BQ-123 (100 nM), but not an ET_B-receptor antagonist, A-192621 (100 nM), blocked ET-1-altered SOCS3 gene expression. The data were normalized for the basal gene expression in vehicle-treated cells. The results of one of two representative experiments are shown. Data are expressed as the mean ± SEM. $^*P < 0.05$.

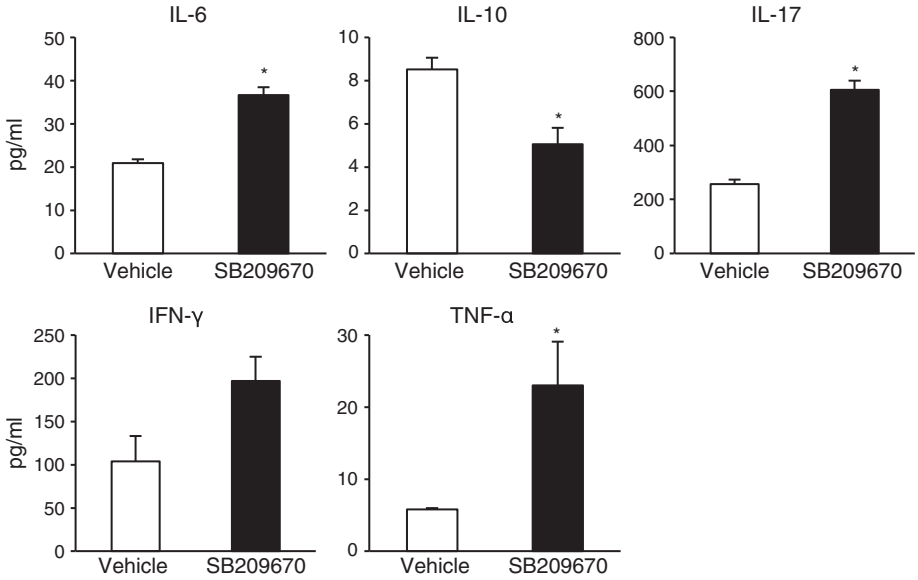

Fig. 6. Flow cytometry analysis of $Foxp3^+$ Tregs in the hearts. The numbers in the outlined areas indicate the percentage of heart-infiltrating $CD4^+$ T cells that express Foxp3. The results of one of two representative experiments are shown. Data are expressed as the mean ± SEM. $^*P < 0.05$.

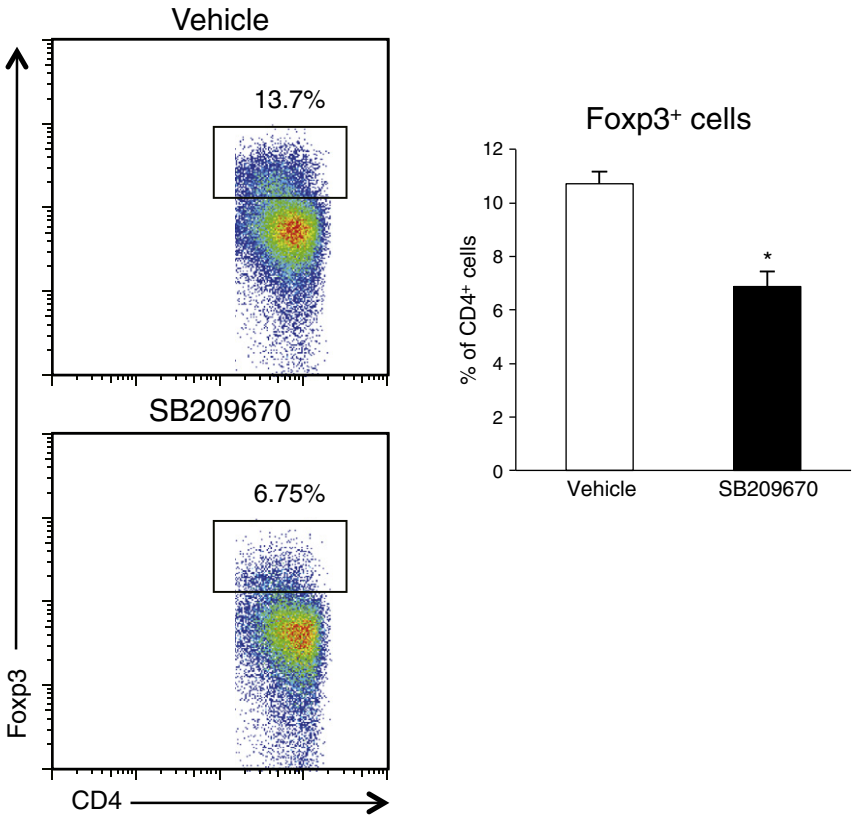

Fig. 7. Cytokine production of cardiac myosin-specific $CD4^+$ T cells. $CD4^+$ T cells were isolated from SB209670- or vehicle-treated EAM mice and cultured with 5 μg/ml of MyHC-α in the presence of irradiated splenocytes for 2 days. The cytokines in the culture supernatant were measured by ELISA. Data are expressed as the mean ± SEM from triplicate culture wells. The data are from 1 of 2 experiments performed, with similar results. $^*P < 0.05$.

Tregs are a subpopulation of T cells which modulate the immune system and express Foxp3, a master regulator in the development and function of Tregs. Tregs are known to play a crucial role in preventing autoimmune disorders and actively controlling autoimmune responses (Shevach et al., 2006; Zheng and Rudensky, 2007). In the SB209670-treated hearts, the $Foxp3^+$ Tregs were more decreased than in the vehicle-treated hearts (Fig. 7).

ET-1 does not have a direct effect on $CD4^+$ T-cell differentiation

Therefore, we wondered whether ET had a direct effect on the T-cell differentiation. To answer this question, we isolated naïve $CD4^+$ T cells from healthy mice and cultured them with ET-1 for 3 days, and then analyzed the T-cell differentiation. ET-1 did not affect the production of the Th1 cytokine IFN-γ, Th17 cytokine IL-17 or Th2 cytokine IL-4. Further, the expression of the Foxp3, a master regulator in the development of Tregs, was not affected by ET-1 (Fig. 8A and B). Thus, ET-1 did not directly influence the T-cell differentiation. We also examined the possibility of a situation in which ET_A- and ET_B-receptor-mediated actions produce opposing effects and interact with each other. ET-1 did not influence the T-cell differentiation in the absence or presence of selective ET_A- or ET_B-receptor antagonists (Fig. 8C). These results may have denied the possibility of a functional antagonism of ET_A-mediated and ET_B-mediated actions.

Discussion

The lymph node is a critical crossroad for encounters between APCs, antigenic substances from lymph and lymphocytes recruited into lymph nodes from the blood (Gretz et al., 1997). Interestingly, various ET-related gene expressions were more dramatically changed in the lymph nodes rather than in the hearts during the course of the EAM (Fig. 1). Notably, ET-1 and ET_A-receptor gene expressions in the lymph nodes were significantly upregulated after the 1st and 2nd MyHC-α immunizations, but not in the hearts (Fig. 1). Thus, the ET-1–ET_A signal may play a key role in the immune responses in the lymph nodes.

EAM is a $CD4^+$ T cell-mediated disease (Eriksson et al., 2003; Fairweather et al., 2001). The initial activation of naive antigen-specific $CD4^+$ T cells occurs in the lymph nodes (Itano and Jenkins, 2003). Therefore, we hypothesized that ET-1 had an effect on the $CD4^+$ T-cell activation in the lymph nodes. To test this hypothesis, we stimulated $CD4^+$ T cells with ET-1 upon TCR stimulation but, unexpectedly, we did not find any direct effect of ET-1 on the $CD4^+$ T-cell differentiation (Fig. 8). Naïve antigen-specific $CD4^+$ T cells are activated by the presentation of peptide-major histocompatibility complex class II (MHC class II) complexes on the APCs in the lymph nodes (Itano and Jenkins, 2003). From these findings, ET-1 in the lymph nodes may influence the APCs, such as DCs that are essential for priming and expanding the $CD4^+$ T cells (Eriksson et al., 2003), and consequently affect the $CD4^+$ T-cell activation. In the present study, we found that ET–ET_A receptor signaling upregulated the SOCS3 gene expression in DCs (Fig. 5B). Similar effects of ET-1 on adipocytes have been reported (Chang et al., 2012). The authors showed that the ET-1 stimulated SOCS3 gene expression in adipocytes through the ET_A-receptor, mitogen-activated protein kinase, Janus kinase and phosphoinositide 3-kinase pathways, independent of the ET_B-receptor pathway. It has been reported that SOCS3-deficient DCs are aberrantly activated and promote Th1/Th17 cell differentiation, indicating that SOCS3 serves to dampen the DC activation and subsequent $CD4^+$ T-cell differentiation (Qin et al., 2012). From these findings, the inhibition of the SOCS3 induction in DCs by ET_A-receptor antagonists may have promoted the T-cell differentiation, which may have resulted in the adverse effects of the SB209680 in EAM. Further studies are needed to evaluate the role of the ET-1 in both innate and adaptive immunities.

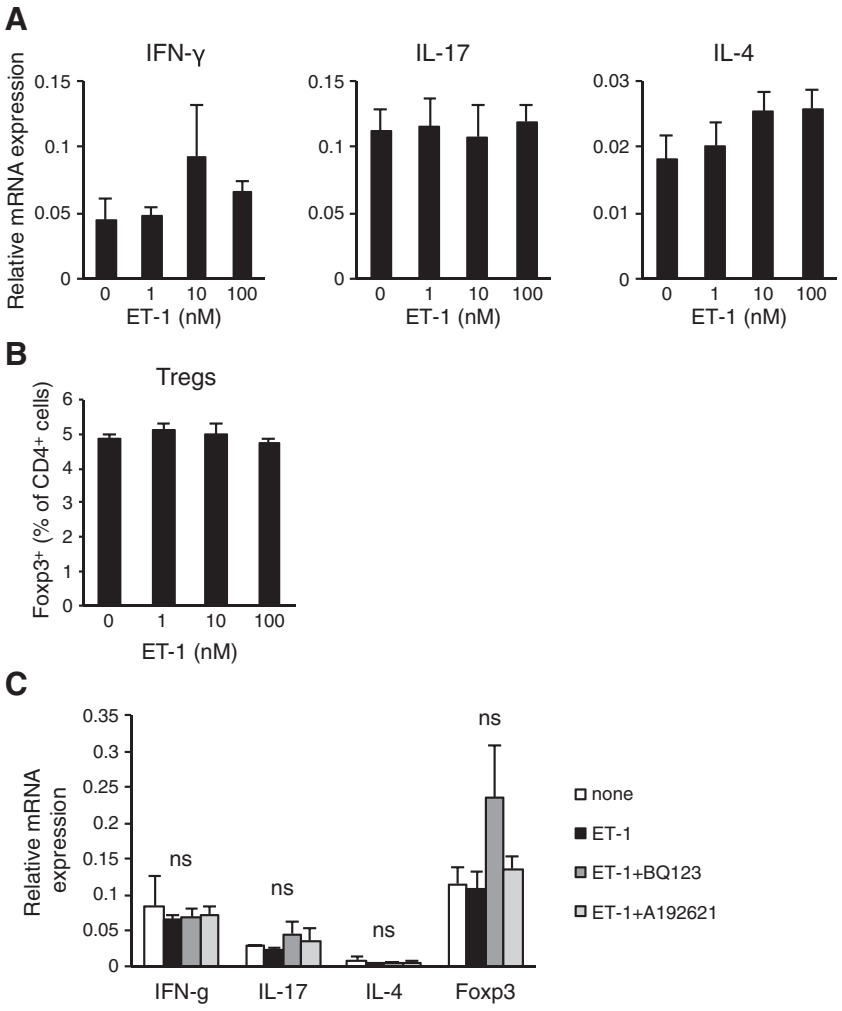

Fig. 8. ET-1 does not influence the $CD4^+$ T-cell differentiation. Purified $CD62L^+$ $CD4^+$ cells (naïve $CD4^+$ T cells) were stimulated with anti-CD3 and anti-CD28 antibodies and ET-1 under Th1-, Th2-, Th17- or Treg polarizing conditions for 48 h. (A) Th1 (IFN-γ), Th2 (IL-4) and Th17 (IL-17A) cytokine mRNA expression in $CD4^+$ T cells was quantified by QRT-PCR. (B) The percentage of $Foxp3^+$ cells was analyzed by flow cytometry. (C) ET-1 (10 nM) did not influence the T-cell differentiation in the absence or presence of selective ET_A- or ET_B-receptor antagonist. Data are expressed as the mean ± SEM from triplicate wells. The results of one of two representative experiments are shown.

The activation and differentiation of T cells play a critical role in the pathogenesis of autoimmune myocarditis (Bergelson et al., 1997). To the best of our knowledge there have been no reports on the effect of ET on T-cell cytokine secretion. Our study examined the effect of an ET receptor blockade on the Th response in EAM. In the present study, in vivo treatment with an ET receptor blocker enhanced the Th17 cytokine IL-17 production from cardiac-specific $CD4^+$ T cells (Fig. 6) and reduced the number of infiltrating Tregs (Fig. 7), which was associated with the disease severity. Th17 cells have been implicated in the pathogenesis of various types of autoimmune diseases (Ghoreschi et al., 2011). Tregs maintain immune homeostasis, prevent autoimmune responses, and control the magnitude and duration of inflammatory responses (Lehtimaki and Lahesmaa, 2013). Caution must be exercised during treatment of autoimmune myocarditis when we use ET receptor blockers, because they may increase the severity of the disease through enhancing Th17 response and reducing the Tregs.

SOCS proteins were originally identified as negative-feedback regulators in cytokine signaling (Dimitriou et al., 2008; Yoshimura et al., 2007). The SOCS family is composed of eight members: cytokine-inducible SRC homology 2 (SH2) domain-containing protein (CIS) and SOCS1 to SOCS7 (Croker et al., 2008; Shuai and Liu, 2003). SOCS3 is a key negative-feedback regulator of gp130, which is a common receptor component of the IL-6 cytokine family (Yajima et al., 2011). It has been reported that cardiac-specific SOCS3 knockout mice developed cardiac dysfunction and ventricular arrhythmias through the unregulated activation of gp130 signaling (Yajima et al., 2011). Although the underline mechanism was unclear, SB209670 treatment downregulated the SOCS3 gene expression in the heart (Fig. 5A). ET may have a protective role in the preventing cardiac dysfunction and arrhythmias through the SOCS3 induction.

There are a few publications on the effects of ET receptor blockers on virus-induced myocarditis models, but to the best of our knowledge, not on EAM. In an encephalomyocarditis virus (EMCV)-induced myocarditis model, treatment with Bosentan, an ET_A/ET_B dual receptor blocker, has been shown to reduce cardiac hypertrophy (Seta et al., 2000), cellular infiltration and myocardial necrosis, but it does not change the virus titers (Ono et al., 1999). Marchant et al. showed that Bosentan improved the echocardiographic data, but enhanced the viral load and myocarditis severity in CVB3-induced myocarditis (Marchant et al., 2009). Thus, antagonism of ET signaling is potentially a desirable therapeutic strategy for cardiac hypertrophy; however it is still unclear whether an ET receptor blockade is useful for controlling the virus-induced myocarditis. In the present study, we evaluated the effect of an ET receptor blockade on myocarditis under infectious pathogen-free conditions, and treatment with an ET receptor blocker exacerbated the disease severity. From these findings, we must be careful when using ET receptor blockers in patients in a state of both virus-induced myocarditis and post-infectious myocarditis.

Conclusion

In conclusion, ET receptor antagonists exacerbated the autoimmune myocarditis in mice. We provide a warning about the use of the ET receptor antagonists during myocarditis.

Conflict of interest statement

The authors declare that they have no conflict of interest.

Acknowledgments

We are grateful to Mr. John Martin for revising this manuscript.

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